# Sustained-release endotoxin. A model for inducing corneal neovascularization.

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# Sustained-Release Endotoxin

## A Model for Inducing Corneal Neovascularization

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**The rabbit corneal pocket assay is one of the most frequently used systems for the study of angiogenesis. This model particularly is useful to identify stimulators of new blood vessel formation. More recently, however, interest in inhibitors of angiogenesis has grown, and several antiangiogenic agents have been identified. Investigations of angiogenesis inhibitors require a reliable model for the stimulation of neovascularization. One method was modified to produce corneal neovascularization by implanting into the rabbit cornea a sustained-release polymer containing endotoxin (*Escherichia coli* lipopolysaccharide). The implant was prepared by mixing weighed quantities of endotoxin with ethylene vinyl acetate copolymer (Elvax) and forming 1-mm$^3$ pellets containing 1%, 7.5%, 15%, 20%, 30%, and 40% (w/w) of endotoxin. Pure Elvax pellets were implanted as controls. Intrastromal corneal pockets were created in 92 eyes of male, albino New Zealand rabbits (n = 80), and sterilized endotoxin–copolymer implants were introduced. The growth rate of new vessels was measured by slit-lamp biomicroscopy. Endotoxin loads of 15% (n = 40) produced a strong neovascularization response with minimal stromal edema, with a mean growth rate of 0.21 ± 0.12 mm/day. Loads of 1%, 7.5%, and 20% yielded 0.1 ± 0.03 mm/day, 0.27 ± 0.05 mm/day, 0.30 ± 0.06 mm/day, respectively (n = 8, each group). Higher loads (30% and 40%; n = 8, each group) produced intense neovascularization, accompanied by severe corneal edema that obscured accurate measurement of the vessels. Corneal pockets that did not contain polymer implants were avascular. When endotoxin–Elvax pellets were removed, the new vessels regressed within 2 weeks. This method offers the advantages of consistency and reproducibility over current models, such as silver nitrate, tumor, silica and thermal burn, for induction of corneal neovascularization and provides a reliable system for the study of angiogenesis inhibitors. Invest Ophthalmol Vis Sci 32:2906–2911, 1991**

Neovascularization is associated with pathologic processes involving virtually every ocular tissue, including proliferative retinopathies, neovascular glaucoma, and corneal neovascularization. However, only a few of these conditions respond well to conventional medical therapies. A proposal advanced in 1971[1] suggested that specific inhibitors of angiogenesis could be used to treat pathologic neovascularization. Numerous inhibitors of angiogenesis were reported[2,3] (including heparin and angiostatic steroids,[4–6] a collagenase inhibitor,[7] platelet factor IV,[3,8] vitreous-derived inhibitors,[9,10] and several synthetic compounds.[11] Moreover, the angiostatic effect of several inhibitors is potentiated by heparin,[5] an arylsulfatase inhibitor,[12] and sulfated beta-cyclodextrins.[13,14]

As these compounds became available in sufficient quantity for large-scale animal studies, the testing of angiostatic drugs to treat ophthalmic diseases became feasible. However, models for the induction of ocular neovascularization are difficult to reproduce and quantify. Previous models of cornea neovascularization include intrastromal implants of syngeneic tumors (V2 and Brown-Pearce carcinoma)[15,16] and silver nitrate,[17] alkali,[18] and thermal burns.[19] The tumor-implant method generates the most powerful neovascular response, but it is limited both by inconsistent and excessively rapid tumor growth after tumor vascularization. Neovascularization induced by silica particle implants is similarly difficult to reproduce. The burn models are based on injury to the corneal surface, also difficult to repeat quantitatively; furthermore, the burns render the cornea susceptible to microbial keratitis and ulceration.

We developed a model using a sustained-release *Escherichia coli* endotoxin implanted into the rabbit

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cornea that offers the advantages of consistency and reproducibility over the previous inducers of corneal neovascularization. This model should be useful for the study of both angiogenesis stimulation and inhibition.

## Materials and Methods

All animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research. Male albino New Zealand rabbits (4.5–6.5 kg) were used in all experiments.

### Preparation of Sustained-Release Polymers

Polymers were prepared as described earlier.[20] Ethylene vinyl acetate copolymer (Elvax; Dupont, Wilmington, DE) beads were washed in high-grade absolute ethanol to spectrophotometric purity. The polymer then was dissolved in methylene chloride at room temperature to a final concentration of 10% (w/v). The quantity of endotoxin required to achieve various percent polymer loading was calculated according to the following equation:

$$\text{Desired percent load} = X/(0.1 + X)$$

where X = mg of endotoxin/ml polymer

A weighed quantity of lyophilized endotoxin (*E. coli* lipopolysaccharide; Sigma, St. Louis, MO) was mixed with the 10% Elvax and vortexed vigorously to produce a homogeneous suspension. An eight-well Teflon mold was prechilled on dry ice, with its working surface covered with a glass plate to prevent condensation in the wells. The endotoxin–Elvax suspension was delivered through a sterile glass pipette and allowed to polymerize for 10 min. The endotoxin–Elvax solid was kept at −20°C for 24 hr to complete polymerization and then desiccated for 48 hr to evaporate residual solvent. Polymer pellets were cut with a scalpel blade into 1 $mm^3$ weighing 1.3 ± 0.3 mg/pellet. Before implantation, polymer pellets were sterilized by ultraviolet irradiation.

### Corneal Pocket Assay

All surgical procedures were done using sterile technique. The rabbits were placed under deep general anesthesia with intravenous pentobarbital (25 mg/kg body weight) delivered through an ear vein. The corneas were anesthetized before proptosis by topical application of 0.5% proparacaine HCl (Alcon, Hormigueros, Puerto Rico). Intrastromal corneal pockets were created by making a 1.5-mm central incision to approximately one half the depth of the cornea (Fig. 1A). Using an iris spatula, an intrastromal pocket was created to within 2 mm of the limbus (Fig. 1B). Preweighed pieces of the polymer were introduced into the pocket with a glass cannula and Teflon trochar (Figs. 1C–D). The eyes were treated with topical erythromycin (Fougera, Melville, NY) and allowed to heal 48 hr postsurgery before observation with a Zeiss slit-lamp stereoscope every 2 days for 10 days and then every 4 days for the subsequent 2 weeks.

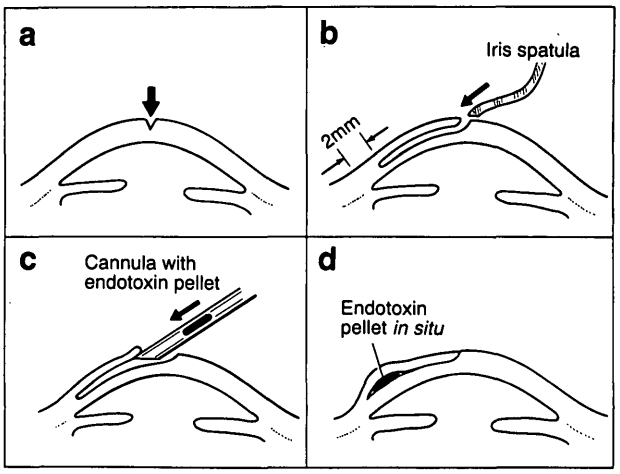


**Fig. 1.** Technique for producing corneal pockets. Pellets of sustained-release polymer containing endotoxin were implanted by: (**a**) making a 1.5-mm central incision to one-half the depth of the cornea; (**b**) using an iris spatula to extend a mid-stromal pocket to a distance of 2 mm from the limbus; (**c**) introducing a preweighed pellet by a glass cannula into the pocket; and (**d**) reapposing the split corneal stroma to allow healing.

For slit-lamp biomicroscopy, the rabbit corneas were anesthetized topically with proparacaine before proptosis. Vessel growth was documented by measuring the longest vessels in millimeters as the perpendicular distance between the limbus and the growing vessel tip. Vessel density and the presence or absence of corneal edema were noted qualitatively for each measurement.

### Histologic Examination

Two weeks after implantation, the animals were killed with an overdose of intravenous pentobarbital, and their corneas were removed and fixed in 10% phosphate-buffered formalin. The corneas were sectioned, then stained with hematoxylin and eosin and Masson-trichrome for histologic analysis of inflammatory cells by light microscopy.

## Results

Initial screening studies of the corneal response to polymers containing endotoxin loads of 15%, 30%, and 40% were conducted. The results of these studies revealed that the 15% endotoxin–polymer implant produced negligible corneal edema that lasted only 3–4 days postsurgery. Higher levels of endotoxin

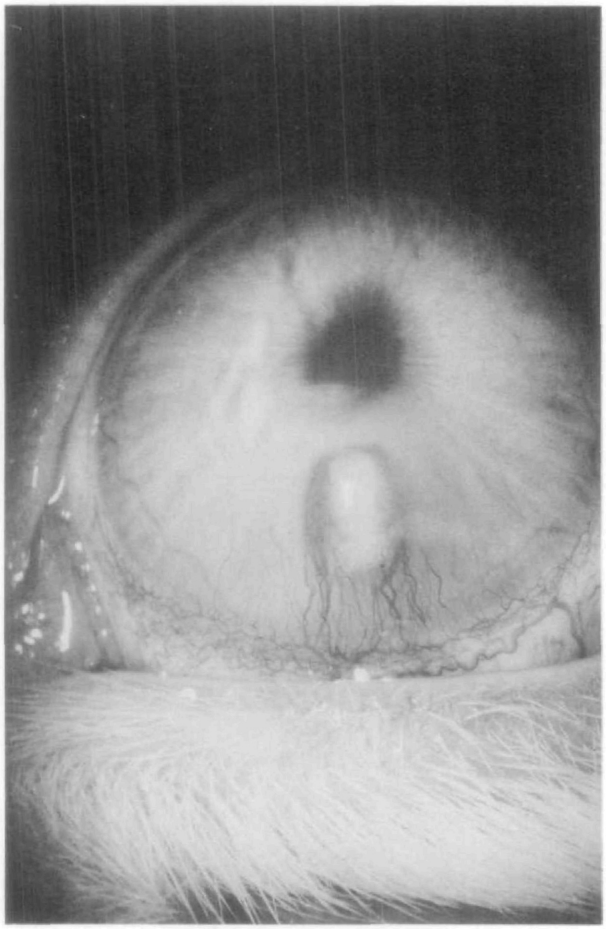

Fig. 2. Neovascular response 9 days after implantation of sustained-release endotoxin (15%) pellet into the rabbit cornea. The endotoxin pellet induced an intense localized neovascular from originating from the limbus and reaching the implant by day 9 post-surgery.

(30% and 40%, n = 16) induced a florid neovascular response accompanied by severe corneal edema within 24 hr after implantation. In response to the 15% endotoxin load, vessels sprouting from the limbus into the cornea were visible by slit-lamp biomicroscopy by day 4 postsurgery. An intense, well-localized frond of vessels converged toward and reached the polymer by day 9 (Fig. 2). Figure 3 shows vessel length as a function of time after implantation (n = 40) of 1.0–1.5 mg of 15% loaded endotoxin–polymer implant. The average growth rate for the first 11 days postsurgery, during which the vessels first reached the polymer, was 0.21 ± 0.12 mm/day.

Histologic examination of the corneas 16 days after implantation of a 15% endotoxin pellet revealed newly formed vessels growing from the limbus toward the pocket, the composition of which varied according to the corneal region. Immediately surrounding the pocket, a densely packed rim of inflammatory cells was present, consisting primarily of heterophilic polymorphonuclear leukocytes (Fig. 4). In the perivascular space of newly formed vessels, the infiltrate was rich in mononuclear cells, with only scattered polymorphonuclear cells present. Polymorphonuclear leukocytes also were found diffusely distributed in the corneal stroma adjacent to the pocket.

To determine if the neovascular response was dose dependent, vascularization stimulated by the endotoxin–Elvax implants (1.3 mg) with loads of 1%, 7.5%, and 20% was assessed (n = 24). Mean daily growth rates during the linear growth phase were: 0.1 ± 0.03 mm/day, 0.27 ± 0.05 mm/day, and 0.30 ± 0.06 mm/day, respectively. Pure Elvax was implanted as a control (n = 6) and induced three to four vessels per cornea in 50% of eyes, with all vessels less than 0.5 mm in length. Pure Elvax pellets in the remaining 50% of controls did not induce neovascularization. The corneas in which pockets were formed but no polymer was implanted were avascular (n = 6).

Slit-lamp examination of corneas from which endotoxin polymers were removed revealed smaller vessels beginning to thin and regressing within 48 hr. Vessels that appeared to be less than 0.5 mm in length and of small caliber regressed the most rapidly and were no longer visible by 1 week after removal of the implant. Larger vessels regressed more slowly and, by 2 weeks after pellet removal, were evident primarily as ghost tracks devoid of erythrocytes.

## Discussion

The implantation of a sustained-release Elvax polymer containing a 15% load of endotoxin into rabbit corneal pockets resulted in well-localized corneal neovascularization, with vessels converging toward the polymer implant in every case. Newly induced vessels

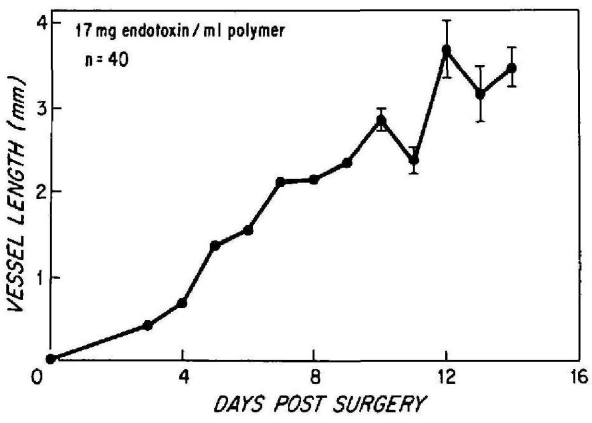


Fig. 3. Time course of vessel growth in response to 15% endotoxin-loaded pellets. Pellets with a 15% load of endotoxin consistently induced corneal neovascularization in rabbit eyes (n = 40), when vessel length was measured by slit-lamp biomicroscopy. All corneas remained clear and compact.

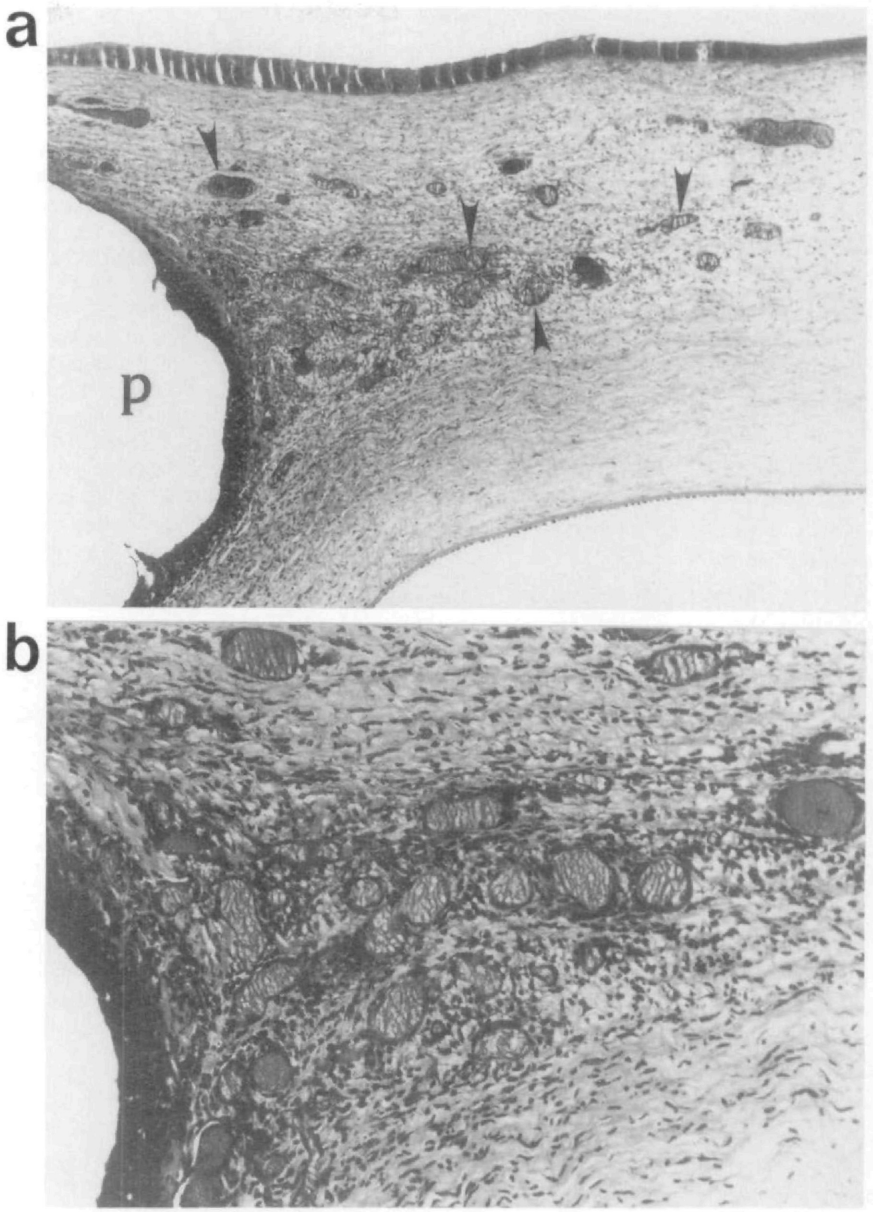


**Fig. 4.** Histologic sections of rabbit corneas 16 days following implantation of an endotoxin (15%) pellet (hematoxylin-eosin stain). (a) New vessels (filled with India ink, see arrows) and an intense inflammatory infiltrate have reached the corneal pocket (P). The implant was removed during tissue sectioning (×16). (b) Detail of above, revealing India ink-filled new blood vessels, mononuclear cells with occasional neutrophils dispersed throughout the stroma, and a densely packed rim of polymorphonuclear leukocytes immediately adjacent to the pocket (×40).

remained in a defined sector of the cornea, and the degree of vascularization was dose dependent. This method offers the advantages of consistency and reproducibility over other animal models for corneal neovascularization such as tumor implants and silver nitrate, silica, thermal, or alkali burns.

We specified maximal angiogenesis with minimal corneal edema as the criteria for the ideal endotoxin load; this was found to be 15%. Loads of 20% or greater produced severe corneal edema, and the resulting stromal opacity precluded accurate slit-lamp measurement of the vessel length. Corneas implanted with polymers containing endotoxin loads less than 15% were clear and compact but had less intense neovascularization. For instance, the rate of neovascularization induced by 7.5% endotoxin loads was greater on average than those induced by 15% endotoxin loads. However, vessel density was significantly greater with the higher endotoxin load. In 50% of the control corneal implants thin, vascular sprouts entered the cornea but did not grow beyond 0.5 mm in length and rapidly regressed within 1 week. Corneas that did not contain any implant were avascular. When the endotoxin stimulus was eliminated by removing the polymer from the pocket, the corneal vessels regressed. This observation was similar to that of others, who reported that removal of a tumor extract from the cornea led to regression of new capillaries, with subsequent monocyte phagocytosis of desquamated capillary endothelial cells.[21]

Our results were consistent with those of previous studies showing that *E. coli* endotoxin incites an inflammatory corneal infiltrate composed of lymphocytes, mononuclear phagocytes, and macrophages.[22,23] It previously was shown that the presence of corneal inflammation is not an absolute prerequisite for corneal neovascularization.[24] However, our results show that the strength of the inflammatory stimulus directly correlated with the degree of the angiogenesis in this model. Thus, this model induces angiogenesis secondary to an inflammatory reaction. Endotoxin-induced neovascularization presumably is mediated by factors such as basic fibroblast growth factor released by macrophages.[25,26] The response may be amplified by other macrophage products including tumor necrosis factor-alpha and transforming growth factor-beta that also stimulate angiogenesis by an inflammatory response.[27-33]

In our studies of angiogenesis inhibitors, we found the difficulty in quantifying corneal neovascularization to be a major obstacle to studying angiostatic compounds for ophthalmic use. In other models, such as the intrastromal tumor implant and central corneal burns, quantitation was difficult because the vascular response was circumferential and not uniform. These limitations restricted the measurement of neovascularization to fixed, flat-mounted preparations of treated corneas that could be evaluated by computer-assisted planimetry or image analysis.[34,35] This useful but tedious method requires killing the animal and thus obviates continuous monitoring of an implant. The endotoxin model addresses this problem because it yields a localized sector of angiogenesis defined by the implant and limbal arc, approximately 10–20% of the total corneal circumference. A consistent degree of neovascularization in this sector allows daily measurement of the longest vessels and comparison of angiogenesis-inhibitor effects between treated corneas and untreated positive control corneas. Blood vessel inhibition and regression therefore can be documented in the living animal and in flat-mounted corneal specimens. Furthermore, the dose dependence of the endotoxin-induced response allows stimulation of both mild and intense corneal neovascularization; this may be used to simulate the spectrum of severity of clinically encountered corneal neovascularization.

**Key words:** polymer, angiogenesis, inflammation, lipopolysaccharide, angiogenesis inhibition

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